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The Role of Carcinoembryonic Antigen-related Cell Adhesion Molecule (CEACAM) Family in Herpes Simplex Virus Type 1 (HSV-1) Viral Entry

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Declaration

I declare that the thesis has been composed by myself, all the sources cited in the thesis have been clearly listed in the references chapter.

I declare that all experiments are my own work.

I declare that the work has not been submitted for any other degree or professional qualification except as specified.

Signature: _____

Date: 2017.01.31

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Contents

Abbreviations

CEACAMs: Carcinoembryonic antigen-related cell adhesion molecules

CHO cell: Chinese Hamster Ovary cell

DMEM: Dulbecco's Modified Eagle's Medium

DMEM/F-12: Dulbecco's Modified Eagle Medium, F-12 Nutrient Mixture

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

ECL: Electrochemiluminescence

FCS: Fetal Calf Serum

GFP: Green fluorescent protein

HeLa cell: Human epithelial cell

HSPG: Heparan sulphate proteoglycan

HSV-1: Herpes simplex virus type 1

HVEM: Herpesvirus entry mediator

NP: Nucleoprotein

IE: Immediate-early

Ig: immunoglobulin

LB medium: Luria-Bertani medium

MOI: Multiplicity of inection

ORF: Open reading frame

PBS: Phosphate Buffered Saline

PEI: Polyethylenimine

PFU: Plaque forming unit

P/S (Pen-Strep): Penicillin and Streptomycin

RPM: Revolutions per minute

siRNA: Small-interfering Ribonucleic acid

TBS: Tris-buffered saline

TNF: Tumor necrosis factor

Y2H: Yeast two-hybrid

293T cell: Human epithelial cell which derived from human embryonic kidney

3-O HS: 3-O sulfated heparan sulfate

1. Abstract

1.1 Professional abstract

Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are the subgroup of the carcinoembryonic antigen family (CEA) which belongs to the immunoglobulin (Ig) superfamily. Herpes simplex virus type 1 (HSV-1) belongs to the subfamily of Alpha-herpesvirinae, which causes the overt infection by contacting with the secretions of an infected individual. Previous studies indicated the interaction between CEACAM proteins and HSV-1 via the siRNA depletion screen and the yeast two-hybrid (Y2H) system. The application of siRNA knockdown screen showed that CEACAM7 as a cellular protein required for viral replication, Y2H found the interaction between CEACAM family and glycoproteins. These results led to our hypothesis that CEACAM protein family may function as the viral entry receptor.

In this project, we carried out several independent experiments in order to confirm the role of CEACAMs in HSV-1 viral entry. Transient overexpression of CEACAMs on HSV-1 viral-permissive cell lines (HeLa and 293T cells) led to a significant increase in viral replication. The interaction between CEACAM proteins and viral glycoproteins (gB, gD, gH) was confirmed by both Luminescence-based mammalian interactome mapping (LUMIER) assay and Co-immunoprecipitation assay. Finally, overexpression of CEACAM on viral non-permissive CHO cells identified the increase of HSV-1 early infection events when co-expressed with human receptors (HVEM or Nectin).

Therefore, in this study, all the results suggested that CEACAM protein family may be a novel class of HSV-1 viral entry receptor.

1.2 Layman's abstract

Herpes simplex virus type 1 (HSV-1) is distributed worldwide, and it is estimated that the risk of getting infection can be as high as 80%-85% for adults. This prevalently contagious virus causes a variety of human diseases when people get acute infection, including oral cold sores, fever, headache, herpes keratitis and genital sores, especially children under 5 years old are susceptible to HSV-1. In addition, HSV-1 infected people may also undergo the latent infection which results in the potential risk that HSV-1 virus may sneak on the body nervous system. Therefore, it is important to understand and investigate the pathway of HSV-1 viral entry into human host cell.

Previous research in our lab has analysed that carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are likely to be a new class of cellular receptor for HSV-1 viral entry. We found that CEACAMs were implicated in HSV-1 based on the previous work. It showed that the depletion of cell surface molecules (including CEACAM7) with deconvoluted small interfering RNA smart pools inhibited HSV-1 replication. Furthermore, the interaction between several CEACAM proteins and HSV-1 viral glycoproteins was confirm as well through a protein-protein interaction screen called yeast two-hybrid.

In this study, several techniques were introduced to further prove our hypothesis and to analyse the role of CEACAM protein family in HSV-1 membrane fusion, infection, gene expression and viral replication. The results from this project indicated that there was an obvious increase of HSV-1 viral replication when CEACAMs were transiently overexpressed into viral-permissive cells. Meanwhile, we confirmed CEACAM proteins could physically interact with HSV-1 viral glycoproteins. Finally, overexpressed CEACAM proteins in viral non-permissive cells enabled to promote the early HSV-1 infection event when combined with other human receptors.

Therefore, our research aims to understand that CEACAM receptor may potentially contribute to HSV-1 viral entry to host cells of human beings.

2. Introduction

2.1 HSV disease

2.1.1 Herpes simplex viruses

There are two subtypes of herpesvirus which are herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), they both belong to the large human herpesviridae family. These DNA viruses are ubiquitous and contagious, both HSV-1 and HSV-2 cause infectious diseases in humans. All herpesviruses have morphological similarity, and on the basis of their biological and virologic properties and DNA sequence homology and genome arrangement, they are classified into three subfamilies, which are Alpha-herpesvirinae (including HSV-1 and HSV-2), Beta-herpesvirinae, and Gamma-herpesvirinae. The defining characteristic of herpesviruses is their ability to establish the latent infection, combing with specific genome of virus into host chromosomes (*Kenneth et al., 2004*).

2.1.2 Epidemiology and pathogenesis

Herpesviruses are highly disseminated in nature, and widely distribute in both developing and developed countries. The most common route of transmission for herpes simplex virus (HSV) is contacting with an infected person who is shedding the virus, or by in contact with contaminated secretions directly. According to the previous clinical report, the contact infection from one anatomical site to another is the primary route of transmission, which indicates that the risk of getting infection can be as high as 80%-85% for adults through this route. Apart from the anatomic involvement, the infection also depends on the individual age and immune status of the host as well as the antigenic virus type. HSV-1 causes cold sores on mouth and lips, gingivostomatitis or ulcerative lesions, the primary infection of HSV-1 usually occurs among very young children, especially the peak of incidence is around 5 years old. In addition, HSV-1 as neuroinvasive virus may become latent and hide in the neurons of human bodies. The major infection of HSV-2 is often postponed until adolescent age and coincides with the sexual activity, therefore genital disease is mainly caused by sexual transmission for both HSV-1 and HSV-2 (*Gupta et al., 2007; Schiffer et al., 2014; Sperling et al., 2008*).

The first step of clinical pathogenesis is primary infection. The manifestation is acute herpetic lesion or whitlow. Saliva, skin blister and infected mucous membrane area are the main modes of infection, the contact triggers the formation of vesicle for non-immune person. Vesicular lesions would continue seven days and viral

shedding lasts at least three weeks. After the primary infection, herpesvirus may conceal in sensory nerve for life long, typically persist in the trigeminal ganglia (HSV-1) and in the sacral ganglia (HSV-2). These non-eradicated herpesviruses come from the latent state cannot be recognised by the immune system and might be reactivated over time, but the mechanism by which recurrence occurs is unknown (*Gupta et al., 2007; Koelle & Corey, 2007*).

2.1.3 Treatment and prevention

The long-term outlook of HSV is that people who get infection will have the virus for the rest of their lives, even though it does not manifest symptoms. After the virus becomes dormant, some people may also experience regular outbreaks with certain stimuli, including stress, fever, menstrual periods, and sun exposure. Treatment always focuses on getting rid of cold sores and limiting outbreaks, medications (such as acyclovir, famciclovir, valacyclovir) help to reduce the intensity and frequency of outbreaks and lower the risk of recurrence.

The obvious way of decreasing the risk of infection is to avoid direct physical contact with lesion. It is also believed that the body may start to create antibodies through immune system over time. If a generally healthy individual has been infected with the virus, there are usually no complications. People can take precautionary measures to avoid becoming infected, or to prevent spreading HSV to another person as well. Meanwhile, share the daily items can also pass the virus around, such as cups, towels, clothing, makeup, or lip balm. It is also recommended that infected individuals should not participate in oral sex, kissing, or any other type of sexual activity, during an outbreak. Additionally, washing hands thoroughly and frequently to reduce the risk. Women who are pregnant and infected may have to take medicine to prevent the virus from infecting their unborn babies (*Corey & Wald, 2009; Kimberlin, 2007*).

2.2 HSV-1 virus

2.2.1 HSV-1 structure

2.2.1.1 Virus structure

All herpesviruses share similar structural properties. The virion size varies from 180 to 300nm due to the thickness of tegument (see below). The structure of HSV-1 contains a large double-strained linear DNA genome inside the core, which the diameter is around 75nm (*Whitley et al., 1996*). The nucleus is established with an

icosahedral capsid, this nucleocapsid is 100-110nm in diameter which has 162 capsomeres. Tegument is the protein-filled amorphous structure between capsid and envelop, this region includes viral enzymes, some of which take control of the cellular chemical process, virion production and defence against immediate response of host cells. Outside, the tegument is encased by a trilaminar lipoprotein membrane called envelope, derived from the cell membrane. Within the envelope is a dozen of virus coded glycoprotein spikes, these glycoproteins offer adherent sites between virus and host cell surface (*Edward, 2003; Smith et al., 2014*) (Fig.1).

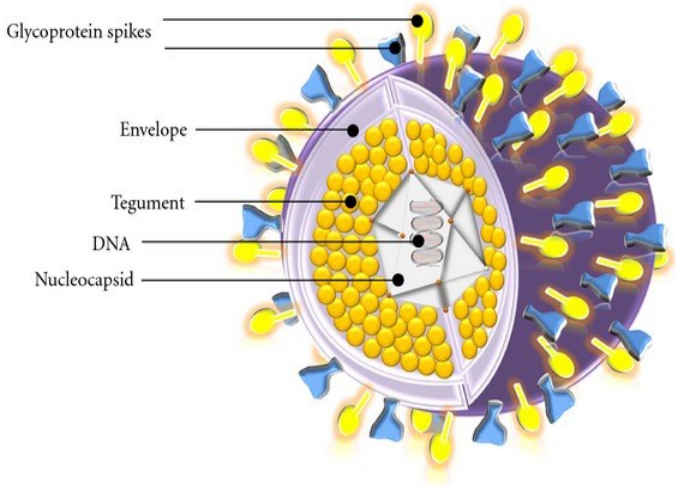


***Figure 1*: HSV-1 virus structure.** The herpesvirus virion consists of five main structural elements. The core contains linear DNA, which is covered with nucleocapsid. Tegument is protein structure between capsid and envelop out layer. Envelop is cellular membrane with glycoprotein spikes on the surface (Taken from http://ukulelejohn.com/tag/structure).

2.2.1.2 Genome structure and organization

The HSV-1 viral genome is a large double-strained linear DNA, the length ranges from 120,000 to 230,000 base pairs with a base composition of 70% G+C, and contains 60 to 120 genes (*Pertel & Spear, 1999*). The genome can be classified into two unique segments, unique long (UL) region and unique short (US) region, both of which encode distinct open reading frames (ORFs). Among the 74 known ORFs, at least 56 viral genes belong to UL, whereas US contains 12 ORFs (*McGeoch DJ, 2006*). The UL is bounded by a terminal repeat long region (TRL) and an inverted internal repeat long region (IRL). The US is similarly bounded by a terminal repeat short region (TRS) and an inverted internal repeat short region (IRS). The inverted repeats allow rearrangement of unique regions, these inverted repeats contain sequences that required for the viral genome packaging or

cleavage (*Salameh et al., 2012*). The long repeat (RL) is about 9,000 base pair, encode immediate early protein and promoter as well as genes for the latency associated transcript (LAT). The short repeat (RS) is about 6,600 base pairs, also encode immediate early protein and work as transcriptional activator to provoke viral gene expression and stimulate viral replication (*Edward, 2003*). There are three different stages of virus genome: linear, circular (after infection), and concatemeric, but the mechanism of genome circularization has not been established (*McGeoch et al., 2006; Mettenleiter et al., 2006*).

2.2.2 HSV-1 viral entry

2.2.2.1 Viral entry routes

The HSV-1 entry mechanism is a multistep process, there are two principle routes of viral entry. The first pathway contains both attachment and penetration, HSV-1 virus attaches to cell membrane results in the interaction between viral glycoprotein and host cell membrane, which allows the entry of viral capsid into host cells. In this pathway, the first step is that glycoproteins gB and gC attach to heparan sulphate proteoglycan (HSPG) on host cell in order to concentrate virus particles on cell surface, which transport is known as viral surfing. The next process is the interaction between cellular receptor and the specific gD receptor of HSV-1, this interaction invades viral fusion to the cell membrane and brings both viral envelop and cell plasma membrane into close juxtaposition (*Campadelli-Fiume et al., 2000*). During the fusion, gD undergoes a conformational change when binging to cellular receptor, which assembly transmit a signal to activate the fusion machinery, gH and gL interact with cell receptors also stimulate the fusion (*Karasneh & Shukla, 2011*). The naked viral capsid is released into cytoplasm after the fusion and the incoming viral nucleocapsid is transported into nucleus, and finally targeted to the nuclear pore where the viral genome is uncoated for viral transcription and replication in the nucleoplasm (*Spear and Longnecker, 2003*). The second mode is that HSV-1 virion

can enter into cells by fusing with cell surface which is called endocytic pathway (Fig.2).

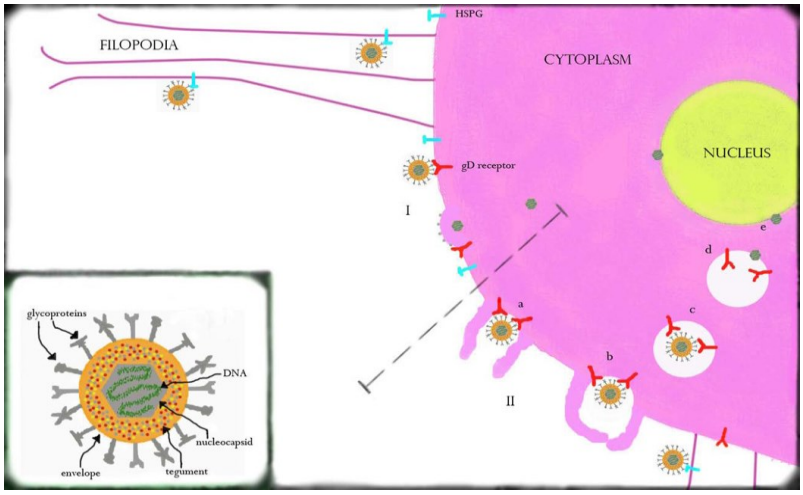


***Figure 2*: Pathway of HSV-1 viral entry into host cell. I**: HSV-1 can attach and fuse with cell surface and enter into the cell cytoplasm. **II**: Endocytosis. a) Virus delivers to the membrane protrusion on cell surface. b) Through the phagocytic function, the virus would enter into cells and towards to the cell nucleus. c) The viral envelope fuses with the membrane. d) The naked viral nucleus capsid is released. e) The subsequent entry into nucleus (*Salameh S, 2012*).

2.2.2.2 Glycoproteins and receptors in the viral entry process

There are four necessary glycoproteins encoded by the HSV-1 genome for the viral fusion which are gB, gD, gH, gL. gB (encoded by UL27) is the principle mediator of initial attachment with HSPG as well as enhance the overall binding of virus particles to cell surface in the initial interaction. gD (encoded by US6) is the major receptor which is able to bind to the specific cellular receptors, including herpesvirus entry mediator (HVEM), nectin-1/-2 and 3-O sulfated heparan sulfate (3-O HS) (*Salameh et al., 2012*). gH (encoded by UL22) is activated followed by gD structural change and in turn regulates activation of gB (*Atanasiu et al., 2010*). gL (encoded by UL1) form a dimer with gH, modulating gH and regulating its activity by ensuring it has been processed correctly and inserted in the membrane (*Gianni et al., 2010*).

HVEM which belongs to tumor necrosis factor (TNF) receptor family is the first gD receptor. Nectin-1/-2 are the members of immunoglobulin superfamily (Ig) and can also mediate viral entry by binding with gD. 3-O HS is another receptor for gD, the

function of 3-O HS is either triggering viral entry or inducing cell fusion (*Karasneh & Shukla, 2011*). The binding between gD and one of these three cognate receptors may lead to the conformational changes of gD, and interact with gB, gH, gL, which together form a fusion active complex.

2.2.3 HSV-1 replication cycle

There are two routes of virus replication, which are productive replication cycle and latent infection. For the former infection, three distinct processes of gene transcription and protein synthesis are initiated when virus entry into the host cell nucleus, which are the immediate-early, early, and late proteins. The cellular DNA repair enzymes may circularise the viral DNA when migrates to nucleus pore. For the latent infection, viral DNA remains linear and inhibits cellular DNA repair or amplify, also there is no viral gene expression for the replication (*Boehmer & Nimonkar, 2003*).

2.2.3.1 Immediate-early gene expression

Immediate-early (IE) gene expression is the first stage of HSV-1 viral transcription after the viral DNA migrates into the cell nucleus, accompanied by some tegument proteins or regulators. VP16 (also known as α-TIF) is presenting in the virion protein and is essential for the expression of the immediate-early genes (*Francisco et al., 2004*). During the immediate-early infection, virion associated VP16 interacts with cellular regulators Oct-1 and host cell factor (HCF), and directly bind to a "TAATGARAT sequences" element in IE enhancers, which then forms a ternary complex. VP16 also stimulates the RNA polymerase II (RNA Pol II) by interacting with mediator components such as TATA box in order to further activate the transcription of immediate-early entry infection (*Triezenberg et al., 1988; Spector et al., 1991; Herrera et al., 2004*). In addition, another regulator is called virion host shutoff (VHS) protein, aims to degrade host mRNA and regulate viral gene expression (*Smibert et al., 1994; Matis & Kudelova, 2001*).

VP16 induces the expression of the immediate-early (IE) transcripts which are ICP4, ICP0, ICP27, ICP22, ICP47. They modulate with VP16 which strongly activate the IE gene expression, and IE genes drive the expression of early transcription. The immediate-early transcription occurs at promoters upon the viral genomes enter the cell nucleus. ICP4 and ICP27 are essentially required for viral replication and transactivation, ICP0 is the crucial factor for paving the way of viral replication and for transacting promoters, the function of ICP22 and ICP47 are

maintaining capability of virus and mediating infection response (*Edward, 2003; Pritchard et al., 2013; Wagner & DeLuca, 2013*).

2.2.3.2 Early and late gene expression

During these two stages of lytic gene expression, protein products can be detected, and proteins produced from early stage such as DNA polymerase (UL30) and DNA binding proteins (UL42, UL29), are then used in the regulation of HSV-1 viral genetic replication. Proteins from late stage are important components for the late kinetics, which applied to package the capsid. DNA replication significantly influences on the viral gene expression, which followed by the late gene expression (*Edward, 2003*).

2.2.3.3 Envelopment and release

There are three different pathways for envelopment, the primary envelopment (at inner nucleus membrane), the de-envelopment (at outer nuclear membrane), and reenvelopment (at trans-Golgi network) (*Leuzinger et al., 2005*).
The primary envelopment occurs at the inner nuclear membrane. The neonatal nuclear capsids migrate into the perinuclear space, during which process tegument and the dense envelope are produced. Then virons transfer to the cell rough endoplasmic reticulum (RER) cisternae from the perinuclear space, and the pre-existing dense envelope helps the transportation of virions from RER to Golgi cisternae where several virions are packed into the large vacuoles (*Leuzinger et al., 2005*). For the de-envelopment, cytoplasmic capsids are formed at outer nuclear membrane. Finally, single virion is released from the vacuole and travel to the cell surface with Golgi-derived vesicles, producing the mature infectious particles and get released from cells by fusing with plasma membrane (*Granzow et al., 2001*).

2.3 CEACAM proteins family

The carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) belong to a subgroup of the carcinoembryonic antigen (CEA) family, which are members of the immunoglobulin (Ig) superfamily (*Zebhasuer et al., 2005*). Each CEACAM plays a precise physiological role as the modulator, such as cell adhesion, cell recognition and regulation, tissue architecture and proliferation, tumor progression and suppression, especially different CEACAMs can mediate cell signal transfection and integrate signal into functional human organs, as well as intersect with other cellular reporters (*Tchoupa et al., 2014*).

The human CEACAM family includes 12 encoded genes (Fig.3). All CEACAM members have the immunoglobulin (Ig)-like domains, the functional Ig fold provides an interface for efficient protein-protein binding region (*Kuespert et al., 2006*). Ig-like domain can be subdivided into Ig variable (IgV) and Ig constant -1/-2 (IgC1/IgC2) domains based on the variability of amino acids sequences long (*Kuespert et al., 2006*). Each CEACAM consists of the specific N-terminal IgV-like domain and IgC2-like domain, the IgC2-like domain is ranging from zero to six numbers of glycosylation sites with either A or B subtype. These surface expressed domains connect with the cell membrane through either the glycosyl phosphatidyl inositol (GPI) anchors or transmembrane (TM) domain (*Tchoupa et al., 2014; Varki et al., 2009*).

All the physiological functions rely on the homophobic and heterophilic interactions among CEACAM IgV-like domains, which makes the intercellular adhesion come true (*Taheri et al., 2000*). Interestingly, some CEACAM members have glycosylated extracellular domains, for examples, CEACAM1 has one extracellular amino-terminal IgV-like domain with 11 isoforms. CAECAM6 also has six extracellular IgC2-like domains followed single IgV-like domain. However, differ from the epithelial CEACAM1 and CEACAM6, there is no homophobic and heterophilic interaction on the extracellular IgV-like domain of CEACAM3, but undergoes bacterial infection (*Kuespert et al., 2006*).

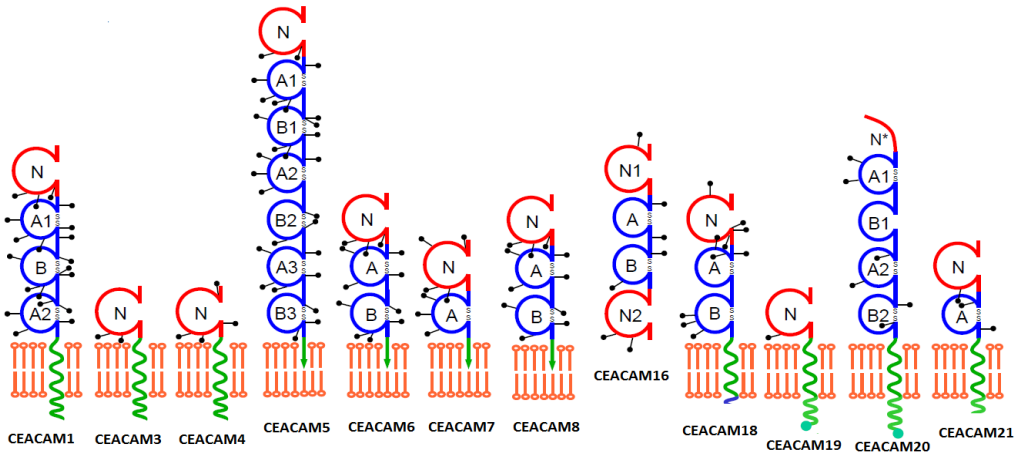


***Figure 3*:** **The members of human CEACAM family.** The red tops are N-terminal IgV-like domains. The blue spheres are 0-6 numbers of IgC2-like domains with A or B subset. Both green arrows and green helices are GPI anchors and TM domains respectively. The black sticks which around each spheres are extracellular domains. Some CEACAMs may have different length of ending cytoplasmic tails, some CEACAMs have distinctive splice variants. CEACAM16 is unknown about the GPI anchors or TM spheres (Taken from http://www.carcinoembryonic-antigen.de).

2.4 Previous work

The CEACAM protein family has already been confirmed to act as cellular receptors of some pathogens for both entry and attachment (*Muenzner et al.,2008*). For example, CEACAM1 is known as the major pathogen receptor for mouse hepatitis virus (MHV) entry which is a murine coronavirus and is also postulated as the receptor for human coronavirus severe acute respiratory syndrome (SARS) (*Hemmila et al., 2004*). CEACAM1, CEACAM3, CEACAM5, CEACAM6 enable to interact with a range of gram-negative bacterials such as Moraxella catarrhalis, Neisseria gonorrhoeae, N. Meningitidis, and Haemophilus influenzae (*Voges et al., 2010*).

The fact that CEACAMs act as receptor for these pathogens led to our hypothesis that CEACAM protein family may be a novel receptor also for HSV-1. Therefore, there were two previous experiments have been done to investigate our hypothesis.

2.4.1 siRNA depletion screen

The small-interfering RNA (siRNA) depletion screen is a powerful technique for phenotypic assay which can be introduced to investigate the virus-host interaction. Previous application of this siRNA depletion screen in our lab has identified the functional role of some cell surface molecules for HSV-1 viral replication.

Briefly, by screening a druggable genome siRNA library, huge amount of human genes was targeted against HSV-1 virus which monitored by the reporter of expressed green fluorescent protein (GFP). This siRNA knockdown screen was carried out into triplicate, one replicate aimed to test the cytotoxic effect of gene depletion through a cell viability assay, whilst other two replicates were infected with HSV-1 virus. The siRNA library was transfected into viral-permissive cell line (HeLa cells) line reversely and then infected with HSV-1, virus growth was recorded by measuring the number of GFP reporter signal (the relative fluorescent units). By calculating the slope from the linear growth carve and comparing with mock-transfected cells control, we identified that some genes (both pro-viral genes and anti-viral genes) influenced HSV-1 growth (*Griffiths et al., 2013*).

A significant reduction of HSV-1 viral replication was revealed when conducted the depletion of several CEACAM members. Specifically, we found the knockdown of CEACAM7 inhibited HSV-1 viral replication, which led to a hypothesis that human CAECAMs might play a role in HSV-1 viral entry receptor.

2.4.2 CEACAM proteins bind to HSV-1 membrane glycoproteins

A genome-scale yeast two-hybrid (Y2H) protein interaction screen was carried out to investigate the infection of viral proteins partner in parallel with siRNA depletion screen. As a result, the interactions were tested between gHΔsp, gD baits and several CEACAMs preys. Specifically, CEACAM8 (in prey) interacted with gHΔsp (in bait) was confirmed, CEACAM19 paired with gD was detected based on the high activity of α-galactosidase.

Therefore, the functional data from siRNA depletion screen and the interaction data from Y2H suggested that CEACAM family may act as a novel class of receptor for HSV-1 viral entry.

3. Hypothesis and aims

The hypothesis is that CEACAM protein family represents a novel class of entry receptor for HSV-1 and they could bind to HSV-1 membrane glycoproteins.

In this project, the main aim is to further investigate the role of CEACAMs in HSV-1 entry and their replications. Specifically, in my project, the aims are:

1) To confirm the phenotype of CEACAMs on HSV replication using gain-of-function over expression experiments;
2) To validate the interactions between CEACAMS and glycoproteins via LUMIER assays and Co-Immunoprecipitation;
3) To investigate whether CEACAMs influence early infection events through luciferase assay.

4. Materials and methods

4.1 Cell lines and virus stock

4.1.1 Cell lines

HeLa cell: The cell type is a human epithelial cell which derived from a cervical carcinoma.

293T cell: The cell type is a human epithelial cell which derived from human embryonic kidney. The parental line of 293T is HEK293, the difference between these two cell lines is that 293T cells contain large "T" antigen transformed which is important to replicate plasmids and to produce sufficient titer.

CHO cell: CHO is an abbreviation for Chinese Hamster Ovary. This laboratory cultured cell line is a good mammalian cell model and frequently used for expression of recombinant proteins. Particularly, CHO cells are non-permissive to HSV-1 infection, because they do not express human receptors (HVEM or Nectin), and they also have post-entry block to HSV-1 viral replication.

4.1.2 Tissue culture technique

DMEM growth medium was prepared with 5% FCS and 1% Pen-Strep (Appendix A), medium and reagents (i.e. PBS, Trypsin) were warmed up in 37°C water bath before use. Cell lines were maintained in the flask (all the milliliter below was based on T75 flask), washed the flask with 10 ml PBS to remove FCS which inhibits activity of trypsin, then 2 ml trypsin was added and incubated 10-15 minutes in 37°C/ 5% CO_2 incubator until all the cells float up, then neutralized the trypsin by adding 8 ml medium, pipetted the medium up and down to make sure the single cell suspension. Transfer the cells and appropriate volume of medium to a new flask, mixed well and put back to the incubator as the next new cell passage. Splitting the cells when the cell confluence gets 70-80%.

Cells were maintained in DMEM/ 5% FCS/ 1% Pen-Step (293T; HeLa; SK-N-SH) or DMEM/F-12/ 5% FCS/ 1% Pen-Step (CHO).

4.1.3 Virus stock

Herpes Simplex Virus type1 (HSV-1) strain C12, which contains GFP as the fluorescent reporter in stock (*Arthur et al, 2001*).

4.2 Isolation of DNA and restriction digest

4.2.1 DNA amplification

For small-scale isolation of DNA from bacteria, an existing glycerol stock of transformed bacteria was streaked on a LB-agar plate (Appendix A) with appropriate antibiotic (Appendix B), and the plate was incubated with the agar on the top at 37°C overnight in a dry incubator. The next day, a single colony was picked using a pipette tip and dropped the tip into a 15 ml centrifuge tube which containing 5ml LB medium with the selective antibiotic, and incubated in an orbital shaker overnight at 200 rpm/37°C. For large-scale isolation of DNA from bacterial, 200 ml LB medium was transferred (Appendix A) with appropriate antibiotic (Appendix B) to a sterile glass conical flask, then inoculated with 500 µl glycerol stock of bacteria culture, and incubated in an orbital shaker overnight at 200 rpm/37°C.

4.2.2 DNA purification

DNA was extracted from small-scale volumes with QIAGEN® Mini-Prep. According to manufacture protocol, 300 µl bacterial culture was mixed with 300 µl 50% glycerol to make a long-term glycerol stock at -20°C. The culture (5 ml) was centrifuged at 4°C for 10 mins at 4000 rpm, and the bacterial pellet was resuspended with 250 µl Buffer P1. Cells were lysed by adding 250 µl Buffer P2 and inverting 6-8 times gently. Then 350 µl Buffer N3 was used to neutralised the lysate, and the lysate was centrifuged for 10 mins at 13000 rpm. The supernatant was transferred to a miniprep column and spun for 1min at 13000 rpm, then the column was washed by adding 750 µl Buffer PE and spun again for 1min at 13000 rpm. Next, the column was left to dry by centrifuging for 1 min at 13000 rpm, then resuspended in 50 µl nuclease-free water, and incubated for 1 min at room temperature. Finally, the volume was centrifuged for 1 min at 13000 rpm to elute the DNA.

DNA was extracted from large-scale volumes with PROMEGA® Midi-Prep. According to manufacture protocol, 300 µl bacterial culture was mixed with 300µl 50% glycerol to make a long-term glycerol stock at -20°C. The culture was centrifuged at 4°C for 10 mins at 4000 rpm (make sure the balance of each bottle pair), and the bacterial pellet was resuspended with 6ml cell resuspension solution. Cells were lysed by adding 6 ml cell lysis solution and inverting the tubes 6-8 times gently, then incubating at room temperature for 3 mins. Then 8 ml cell neutralisation solution was used to neutralised the lysate, and the supernatant was centrifuged for 30 mins at 4000 rpm. The columns were assembled on the vacuum manifold, and the supernatant was poured into the top of the column and apply vacuum. Then

the white filter (DNA binding filter) was washed by adding 5 ml Endotoxin Removal Buffer and washed again. The column filter was left to dry for several minutes, then 600 µl nuclease-free water was overlaid on the column filter, applied the vacuum again to elute the DNA. The NanoDrop® Spectrophotometer was used to measure the DNA concentration (ng/µl).

4.2.3 Restriction digest and gel electrophoresis

Restriction enzyme digestion was used to identify the correct plasmid DNA. Reagents in the reaction mix included 2 µl DNA sample, 2 µl fast digest green buffer (10X), 2 µl 10%BSA, 0.2 µl restriction enzyme (the enzymes were used depended on the DNA plasmid we choose) to a total volume of 20 µl with ultrapure water (pyrogen-free, DNase-free, RNase-free), incubated the mix at 37℃ for 10 min. The agarose gel was made by dissolving 1% agarose powder into TAE (1X) buffer using a microwave oven, then added 2 µl of SYBR® safe DNA gel stain per 100 ml gel after the gel had cooled down. The gel was then poured into a chamber with a comb so that wells could be created. 8 µl of 1KB GeneRular ladder (MBI Fermentas®) was added into the first well, then the reaction samples were transferred into each well of agarose gel in order. The gel was run at an appropriate voltage for approximately 45-60 mins to separate DNA fragments by size as well as to visualise the DNA isolation and purification. DNA binds were analysed on a short-wave UV transilluminator (Bio-Rad® Gel Doc™ 1000).

4.3 CEACAM overexpression and HSV-1 infection assay

Cells were seeded at 1.5 x 10^4 cells/well for HeLa cells or 2.0 x 10^4 cells/well for 293T cells in 96-well black plate, in a total volume of 100 µl each well. The plate was incubated in the 37°C/5% CO_2 humidified incubator overnight. The next day, pCR3-CEACAM genes were diluted in sterile water to 100 ng/µl. The transfection reaction included 1 µl DNA, 18.8 µl OPTIMEM Reduced Serum Medium (Gibco® by Life Technologies™) and 0.2 µl Lipofectamine Reagent® 2000 (Invitrogen®) was incubated at room temperature for 10-15 min. The media was removed and replaced with 80 µl DMEM medium (no pen-strep) each well. 20 µl the reaction mix was transferred into each appropriate well. The plate was placed in the 37°C/5% CO_2 humidified incubator. After 24 hrs, HSV-1 virus stock was thawed rapidly in a 37℃ water bath and diluted to a multiplicity of infection (MOI) 0.5. The medium was removed from plate, and either added 25 µl diluted virus or 25 µl (uninfected control) in triplicate and incubated 1 hr at 37°C, then replaced with 100µl phenol red-free

DMEM medium in each well. After 22 hrs of post infection, the viral growth was measured every 2 hrs by fluorescence in a plate reader (BMG LABTECH®).

4.4 LUMIER assay

Luminescence-based mammalian interactome mapping (LUMIER) assays were carried out to detect protein-protein interactions. It simplified the protein-protein interaction test significantly due to the direction range of luciferase reporter is sensitive and highly dynamic (Fig.4).

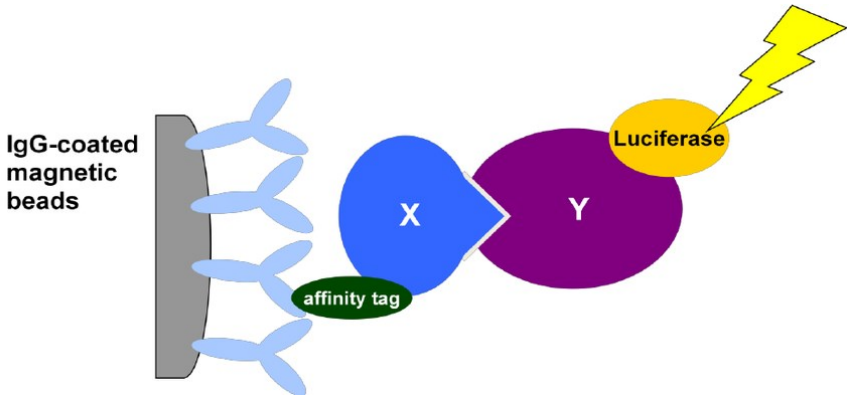


***Figure 4*: The principle of LUMIER assays.** Two proteins are transiently expressed into mammalian cells, after lysing cells, the affinity tag is used for affinity purification of protein X on IgG-coated magnetic beads, the luciferase tag is used for detection of protein Y (*Blasche & Koegl, 2013*).

293T cells were seeded at a density of 1.5 x 10^4 cells/100µl/well in 96-well plate, after incubating at 37°C for 24 hrs, the cell confluence was 60-70% approximately. Then the transfection complexes were prepared as a total volume of triplicate. For one single well of 96-well plate, the mix contained 25 ng pLUM DNA (DNA dilution: 50 ng/µl), 25 ng pTREX DNA (DNA dilution: 50 ng/µl), 0.2 µl Lipofectamine Reagent® 2000 (Invitrogen®) reagent and 18.8 µl OPTIMEM Reduced Serum Medium (Gibco® by Life Technologies™). he complexes were incubated for 10-15 min at room temperature, removed medium from 293T cells plate and added 80 µl of no pen-strep growth medium to each well instead, then carefully added 20 µl transfection mix to each well and returned the plate to incubator. After 48 hrs, the transfected cells were lysed with 10 µl cell lysis buffer (Appendix A) per well which contained the washed Dynabeads (M-280 sheep anti-rabbit IgG, Novex® by Life Technologies™). Cells were lysed on ice for 30 mins or put in the freezer instead until use. The substrate for luciferase measurement was prepared in a 50ml tube

(Appendix A) and covered with foil for at least 30 mins at room temperature. Then the luciferase activity was measured by separating the lysed cells into two plates. Background binding plate (activity plate) contained 90 µl PBS (1X)/well in a black-white 96-well plate, then added 10 µl of each sample/well, leave 5mins at room temperature before measuring. The activity plate gave the background luciferase activity in lysed samples. For the specific binding plate, the remaining cell lysate was transferred to another black-white 96-well plate, held on magnet for 1 min, and carefully remove the supernatant. The plate was washed with PBS (1X)/1mM DTT buffer 4-6 times, then replaced with 100 µl PBS(1X) per well (slow and careful wash so as not to lose beads). The specific binding plate showed the proteins bound to the beads only, but not the background binding of proteins to the beads (see below). Finally, luciferase activity was measured with plate reader (BMG LABTECH®) by adding 70 µl substrate (Appendix A) per well.

Furthermore, in our experiment, we measured four values of each sample: background binding (input), specific binding (bound), and negative controls for both of the ‘’input’’ and ‘’bound’’. Then we calculated the endpoints using the formula: log(specific) / log(background) - log(specific negative control) / log(background negative control), and normalized z-score by subtracting the mean from each value and dividing by the standard deviation.

4.5 Early entry experiments

CHO cells were seeded at 1.5 x 10^4/100µl per well as required in 96-well plate and incubated overnight at 37°C. The transfection reaction was prepared, each well included 60 ng empty pCR3, 60 ng HVEM/Nectin (100 ng/µl), 40 ng ICP4 or ICP27 promoter (100 ng/µl), 18.75 µl OPTIMEM and 0.25 µl Lipofectamine® 2000 reagent. Incubated the reaction 15 mins at room temperature, replaced the CHO cells plate with 80 µl no antibiotics growth medium per well. Then added 20 µl reaction mix to each appropriate well, incubated in the at 37°C/5% CO_2 humidified incubator. After 24 hours, infected cells with 25 µl of DMEM/F-12 infection medium with HSV-1 C12 at MOI 5 dilution, or with 25 µl growth media only as uninfected control. For synchronous infection, plate was put on ice for 1 hr, then transferred into 37°C/5% CO_2 incubator for 1hr. Bound virus was removed by acid wash cells with sterile citrate buffer (Appendix A), 50 µl buffer was added per well for 1min before washing 3-4 times with 100 µl/well of PBS, and replacing with 100 µl/well growth media, then returned to incubator. After 8hrs, the medium was removed and cells were lysed by adding 30 µl 1X Passive Lysis Buffer (Appendix A) to each well,

covered the plate with an adhesive seal and placed on ice for 30 mins. Cell lysates were transferred into a black 96-well plate with inner white well. 30 µl luciferase regent (light sensitive) was added to each well before luciferase activity was measued.

4.6 Co-Immunoprecipitation assay

293T cells were seeded at 5 x 10^6 cells per 10 cm dishes in 10 ml growth medium and incubated at 37°C overnight. The next day, the media was removed from the dish and vaccinia virus was diluted at an MOI of 10 in DMEM medium (without serum or antibiotics), then cells were infected and incubated for 1 hr. Then transfection reaction was prepared per dish, including 4 µg of each required DNA and 24 µl Polyethylenimine (PEI) and 950 µl OPTIMEM, and mixed in one 1.5 ml eppendorf tube, incubated for 30 min at room temperature. All viral inoculum was discarded from dish and replaced with the transfection reaction dropwise and 9ml no pen-strep/5%FCS media followed by incubating in the 37°C/5% CO_2 incubator. After 48 hours, cells were detached by pipetting media up and down and transferred into a centrifuge tube. The supernatant was discarded after centrifuging, and cells were washed in 10 ml PBS and centrifuged again to remove the supernatant. 1.5 ml NP40 buffer (Appendix A) was added to the cell pallet and lysed cells on ice for 30 mins or in the freezer. Then the pallet cells were centrifuged and the supernatant was transferred to a clean 1.5 ml eppendorf tube. Protein amounts in each sample were quantified using the Pierce BCA assay Kit (Thermo®) as per instruction using a clear 96-well plate. According to the manufacture protocol, BCA working reagent was prepared (A: B=50:1) and added 200 µl/well, then 25 µl of each standard (A to I) or 25 µl sample was added to each appropriate well, incubated 30 mins at 37℃. Finally, the absorbance (near 562nm) was measured on the plate reader (BMG LABTECH®) after the plate was cooled to room temperature.

The total volume of beads needed for either pre-clearing (25 µl beads per sample) or antibody-specific (40 µl beads per sample) binding was calculated as required. Beads (Protein G Sepharose™ 4 Fast Flow) were washed 3 times with 1 ml NP40 buffer (Appendix A) before replaced with the initial volume of NP40 buffer. Then 25 µl of washed beads were added to the cell supernatant to pre-clear samples. 5 µl of anti-Myc antibody (Mouse monoclonal IgG, Santa Cruz Biotechnology™) was added to pre-coat beads per sample. Both pre-clearing beads and antibody-coated beads were incubated at 4°C for 1 hr in an overhead rotator. Then beads were centrifuged and resuspended with antibody-coating beads and an initial volume of

NP40 buffer (5 µl per sample). Finally, 40 µl of antibody-coated beads were added into each labelled new tubes, the needed volume of pre-clearing sample was calculated as 200 ug of each protein, and the accurate volume of supernatant was added to each appropriate tube. Then samples were incubated overnight at 4°C in an overhead rotator.

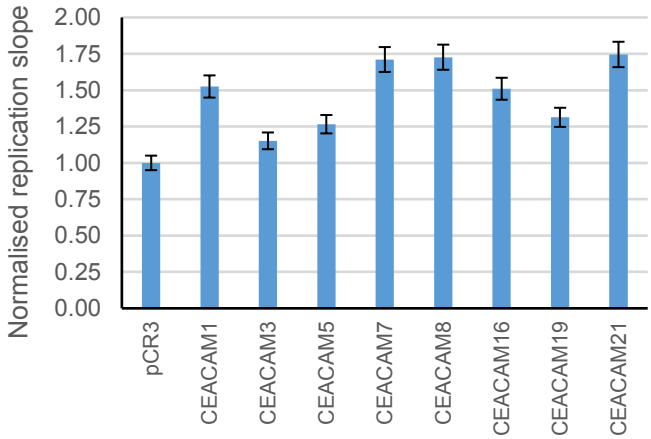
For western blot, the resolving gel (Appendix A) was overlaid at the bottom and the stacking gel (Appendix A) on the top of the back plate. The comb was inserted quickly without air bubbles around the teeth, left until polymerise. The casting apparatus was then moved to the gel running tank which filled with running buffer (1X) (Appendix A). The samples were transferred alongside to each well plus the ladder (Spectra™ multicolour broad range ladder) in the first well. Running the gel until the last ladder band below the weight of protein was just at the bottom of gel. The proteins were transferred from gel to nitrocellulose membrane by semi-dry transfer, then rinsed in TBST (1X) (Appendix A). The membrane was blocked with 5% milk in TBST (Appendix A) overnight. The next day, the membrane was incubated with diluted primary antibody (Rat α-HA) (see Appendix B for the antibody dilution) for 1hr at room temperature on the shaker after the block milk was removed. After 1hr, washed the membrane three times with TBST on the shaker for 30 min in total. Then the diluted secondary antibody (α-rat-HRP) (see Appendix B for the antibody dilution) was added to incubate the membrane for another 1hr on the shaker, repeated washing and rinsed the membrane in PBS (1X) (Appendix A) for 5 min. 3 ml Electrochemiluminescence (ECL) developing substrate (Pierce™ ECL Western Blotting Substrate, Thermo Scientific™) was overlaid on the membrane for 5 min, then put the membrane into development cassette after the ECL was removed. Finally, the photo film (Kodak®) was exposed with different time points to the strength of antibody.

5. Results

5.1 Overexpression of CEACAM proteins increases susceptibility to HSV-1 infection

In order to investigate whether CEACAM proteins could increase HSV-1 viral replication, we used gain-of-function overexpression experiments. HeLa or 293T cells were transiently transfected with either pCR3 vector or CEACAM plasmids before infected with HSV-1 virus, the green fluorescence protein (GFP) was working as a function of the fluorescence reporter, and the HSV-1 viral replication was measured after 22-24 hrs post-infection until 44-46 hrs by detecting the level of GFP signal. In comparison to the fluorescence between CEACAM expressing cells and control cells (with pCR3 only), then the slop was normalised by analysing CEACAMs against pCR3 from replication growth carve. The charts below show the summary of several times repeating of this experiment in each cell line without outliers (Fig.5). Therefore, we could confirm that CEACAMs overexpression had a positive effect on HSV-1 viral replication. Specifically, overexpression of CEACAM7 and CEACAM8 increased viral replication by approximately twofold.

A)



B)

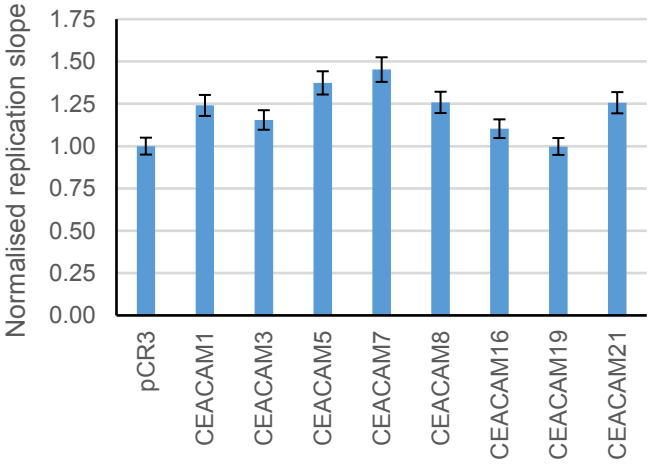
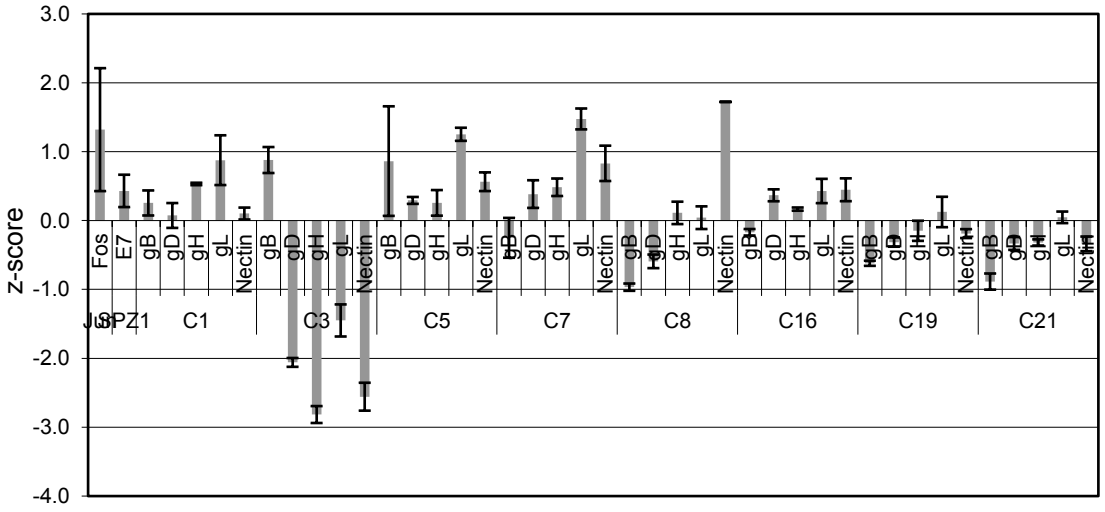


***Figure 5*:** **Overexpressed CEACAM proteins increased HSV-1 viral replication prominently. A)** HeLa cells (seeded at $1.5x10^4$ cells in per 100µl well) were infected with HSV-1(-GFP) at MOI 0.5 after transfected with either pCR3 or CEACAM proteins, the viral replication was measured every two hours after 22hrs post infection until 44hrs. **B)** 293T cells (seeded at $2.0x10^4$ cells in per 100µl well) were infected with HSV-1(-GFP) at MOI 0.5 after transfected with either pCR3 or CEACAM proteins, the viral replication was measured every two hours after 24hrs post infection until 46hrs. The pCR3 worked as background control, the error bars showed the standard deviation based on the triplicates of each CEACAM.

5.2 Confirmation of the interaction between CEACAM proteins and HSV-1 viral glycoproteins

As the interaction between HSV-1 viral glycoprotein and CEACAMs had been previously identified via Y2H system (see "introduction 2.3.2"), luminescence-based mammalian interactome mapping (LUMIER) assays were carried out to confirm these interactions specifically in mammalian cells. Briefly, CEACAMs and glycoproteins were in pLUM and pTREX vectors respectively with both vector directions (CEACAM-pLUM/glycopretein-pTREX; CEACAM-pTREX/glycopretein-pLUM). The charts below show the summary of five times independent repeating. The result identified CEACAMs could interact with glycoproteins on both vector directions (Fig.6). It can be seen that all the positive bars indicated the interaction between CEACAMs and glycoproteins. gB (in LUM) interacted with CEACAM5, CEACAM8, CEACAM16 and CEACAM19, gD (in LUM) also interacted with CEACAM8, CEACAM16 and CEACAM19. Particularly, the strong interaction happened between CEACAMs and gH from the second figure.

A)



B)

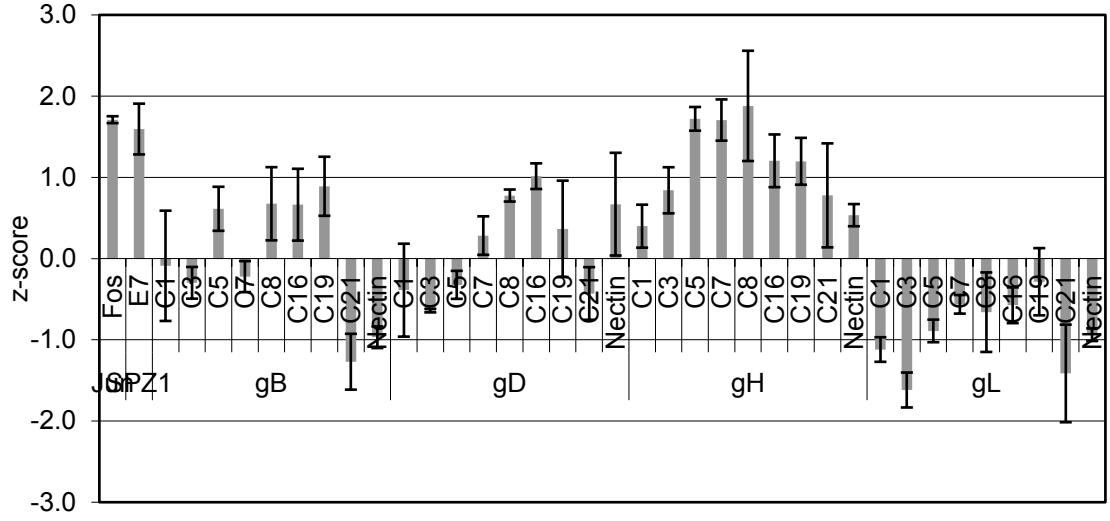


Figure 6: **The interaction between CEACAMs and glycoproteins was confirmed.** Both CEACAM and glycoprotein were transiently transfected into each appropriate 293T cell well (seeded at $2.0x10^4$ cells in per 100µl well). After 48hrs, cells were lysed with buffer contained magnetic beads. Then luciferase activity was measured with lysed samples by the separate background binding plate and specific binding plate. **A)** The interaction between CEACAM (in pLUM) and glycoprotein (in pTREX). **B)** The interaction between CEACAM (in pTREX) and glycoprotein (in pLUM). The error bars showed the standard deviation based on the triplicates of each CEACAM and glycoprotein interaction pair. Two pairs of proteins (JUN&FOS and SPZ1&E7) known to interact were used as positive control, the additional control was each gene of interest (CEACAM or glycoprotein) in pLUM was tested against protein A in pTREX in order to determine any false-positive interaction (this control pair didn't show on the figure). (C=CEACAM, g=glycoprotein)

5.3 CEACAM7 can bind to glycoprotein H (gH) is proved through Co-Immunoprecipitation

In order to further validate the interaction between CEACAM proteins and gH (revealed in the previous LUMIER result), we used a second approach to test this proteins interaction which was co-immunoprecipitation. The recognised protein (antigen) is termed bait, which enable to catch prey protein. CEACAM proteins were attached with the bait protein and gH in prey, bait had MYC tag and prey had HA tag. Reversely, gH in bait and CEACAMs in prey.

In this experiment, after the interaction took place, it can be seen that the bind of CEACAM7 appeared which expected bind size was 30kDa. Particularly, the

intensity of CEACAM7/gH bind (the red square on left side) was much higher than the CEACAM7/empty-bait bind (the red square on right side), which confirmed the interaction between CEACAM7 and gH (Fig.7).

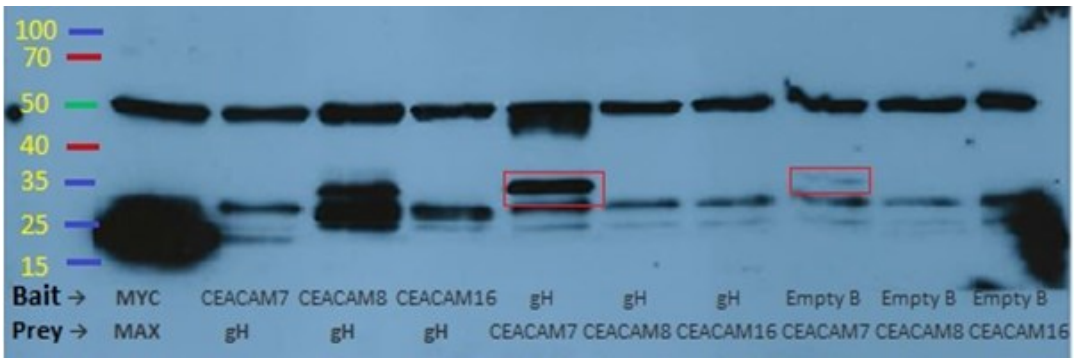


***Figure 7*: CEACAM7 was detected to bind with gH.** Both CEACAM7 and gH were transiently transfected into each appropriate 293T cells (seeded at $5.0x10^6$ cells in per 10ml dish) before infected with vaccinia virus at MOI 10. Cells were lysed after 48hrs and pre-cleared with sepharose beads, followed by pulling down with anti-MYC (α-MYC) antibody coated beads. Then the antibody coated samples were separated based on the weight of proteins via western blot and transferred to the nitrocellulose membrane. The primary antibody (anti-HA or α-HA) and secondary antibody (α-rat-HRP) were introduced to stain the membrane after the membrane had been blocked. Finally, the membrane was developed and the X-ray film was exposed for a appropriate length of time. The film above showed the 10-second exposure. Empty bait group with CEACAM7 (in prey) worked as the background (reference value) in comparison to the gH (in bait)/CEACAM7 (in prey) pairs.

5.4 CEACAM proteins promote early HSV-1 infection events

Given the ubiquitous expression of the known HSV entry receptors, it can be experimentally challenging to demonstrate a novel protein is acting as a receptor. One way can be done is to express potential receptors on viral non-permissive cell lines (such as CHO cells).

We used the luciferase assay to determine whether members of the CEACAM protein family act as a receptor for HSV-1 viral early entry infection. As presented in the introduction section, the VP16 protein which is in the tegument of the HSV-1 viral particle is a transcription factor. When the virus enters into CHO cells, VP16 can bind to the promoter DNA sequence and drive gene transcription, specifically the very first stage is the binding with the immediate-early (IE) gene promoter. In our lab, we have gene promoter (ICP27) which offers the region for VP16 to bind, driving a luciferase reporter. CEACAM proteins have been constitutively

overexpressed into CHO cell lines, CHO cells were non-permissive for HSV-1 so that the expected increase of infection would validate the role of CEACAM as receptors.

The results indicated the significant increase of all the infected CHO-CEACAMs transfected with HVEM, also the infection enhanced for CEACAM3, CEACAM5, CEACAM16 and CEACAM19 with Nectin, which demonstrated that CEACAM enabled to work as the co-receptor with either HVEM or Nectin (Fig.8).

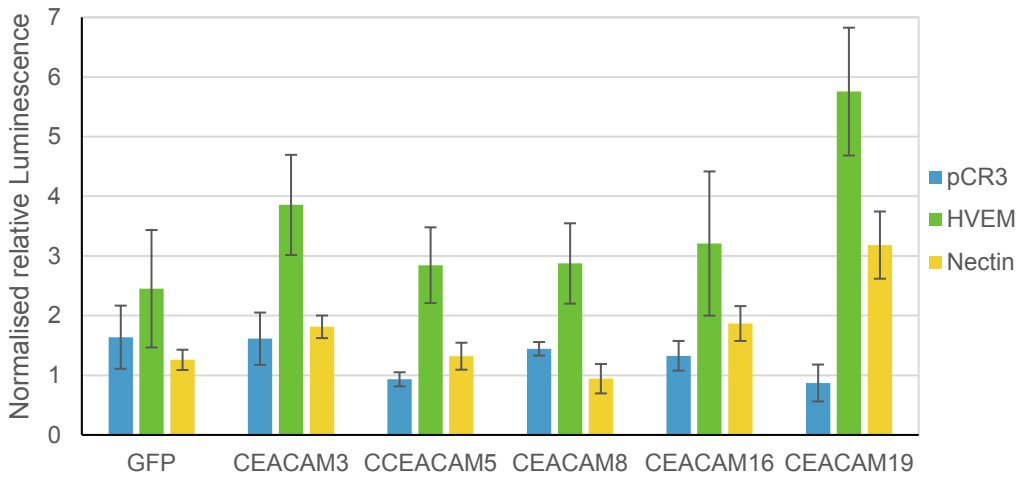


***Figure 8*: CEACAM proteins have a positive influence on HSV-1 early entry.** CHO cells (seeded at $1.5x10^4$ cells in per 100µl well) were infected with HSV-1(-GFP) at an MOI of 5 after transiently transfected with ICP27 gene and either pCR3 or HVEM/Nectin, cells were lysed after 8hrs post infection and the luciferase activity was measured. For data analysis, non-infected CHO-CEACAM cells with pCR3 was the background control (the no virus pCR3 bar didn't show on the figure), and calculated the average of each CEACAM triplicates and normalised against the average of CEACAM-pCR3 cells (no virus) individually, finally calculated the mean and standard deviation of each normalised CHO-CEACAM. The error bars showed the standard deviation based on the triplicates of each CEACAM.

6. Discussion

6.1 Result analysis

6.1.1 HSV-1 viral replication increase prominently with overexpressed CEACAMs

CEACAM proteins were overexpressed based on the gain-of-function overexpression experiment, and the obvious increase of HSV-1 viral replication were detected after 22-24 hrs post-infection. The two bar charts showed the increase level in both HeLa cells and 293T cells. All the CEACAM bars were enhanced compared with the pCR3 control, especially CEACAM7, CEACAM8 and CEACAM21 increased approximately 60% in HeLa cells, and CEACAM5, CEACAM7 also presented the much higher viral replication in 293T cells. The error bars from each triplicate group may due to the confluence of cells in each cell well or the inequality of virus overlay.

6.1.2 Protein-protein interaction through LUMIER and Co-IP

The interaction between CEACAMs and glycoproteins was confirmed via LUMIER assay. It seems that CEACAM7 and CEACAM 8 interacted better with both gB and gD than other CEACAM proteins, and also the interaction between CEACAM7/CEACAM 8 and gB/gD were much stable than other CEACAM proteins from five independent experiments. Whether this phenomenon is based on the phenotype or structure of CEACAM proteins is uncertain. For example, CEACAM16 has special structure which might not contain GPI anchor or TM domain (see “Introduction 2.4.1”) to connect with cell membrane, but CEACAM16 enables to interact with either gB or gD as well.

Furthermore, the interaction between CEACAMs and gH was obvious through the LUMIER assay. However, this result was completely against the previous studies which indicated that the receptor interacted with gH was unidentified (*Campadelli-Fiume et al.,2012*).

Co-IP experiment was carried out in order to further prove that CEACAMs enable to interact with gH. As the result, the expected bind of CEACAM7 was shown with the molecular weight of 30kDa, and the bind intensity was much higher in CEACAM7 with gH than in CEACAM7 with empty bait, which convinced the possibility of the interaction between CEACAM7 and gH.

There were some limitations on both LUMIER assay and Co-IP. From the LUMIER side, the Dynabeads were inevitably lost by repeat washing 3-4 times even though

beads were sucked down to the magic. From the Co-IP side, the concentration of lysed samples influences the volume of needed supernatant for quantifying the proteins and coating the antibody. Meanwhile, other factors such as the cell confluence, the efficiency of transfection, infection rate may also affect the results. In order to minimise the errors, each experiment was repeated 3-5 times independently and each protein-protein pair was in triplicates.

6.2 Interaction between CEACAM proteins and gB, gD, gH, gL

The first stage of HSV-1 entry process is the fusion between the virion envelope and a cellular membrane. The entry fusion machinery is influenced by a number of determinant factors. In terms of the virus, the major glycoproteins (gB, gD, gH, gL) have their specific roles which contribute to the virus-cell fusion. From the cell side of view, a plethora of cellular receptors and the routing factors play the key role on entry pathway.

With respect to the function of viral glycoproteins, the fusion activation begins with one glycoprotein and transmits to another, all the glycoproteins have mutual influence. Initially, gD interacts with one cellular receptor, the binding forms a complex of gD and receptor and also results in a conformational change of gD. This assembly enables the signal transmit to gH/gL as well as contributing to the activation of gB (Fig.9) (*Cooper & Heldwein, 2015*).

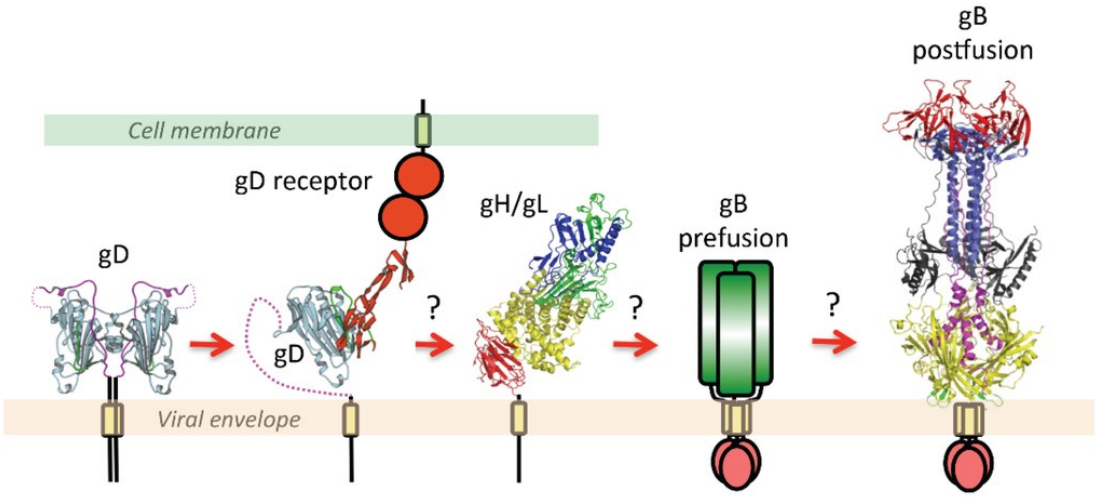


***Figure 9*: The function of gB/gD/gH/gL in fusion process.** The initial interaction happens between cellular receptor and gD on the cell membrane, which triggering a conformational change of gD. gH/gL are activated followed by gD structural change and in turn regulates activation of gB for its prefusion. (Taken from http://www.mdpi.com)

In recent years of studies, several receptors for glycoproteins have been identified. As some researches indicated, there are three main cellular receptors for gD, which are HVEM, Nectin1/2 and 3-OS HS (see “Introduction 2.2.2.2”). The current known receptor for gB is called paired immunoglobulin-like type 2 receptor-a (PILRα) with activating function (*Satoh et al., 2008*). Another receptor for gB is Myelin-associated glycoprotein (MAG) which has approximately 5%-12% homology with PILRα (*Karasneh et al., 2011*). Currently, the only confirmed routing factor of HSV-1 entry for gH is αVβ-intergrin, but whether there is an interaction between the αVβ-intergrin and the component of virion particles is unknown (*Campadelli-Fiume et al., 2012; Gianni et al., 2010a; Gianni et al., 2010b*).

The previous work in Prof. Juergen’s group has shown that some CEACAM members were capable of inhibiting the HSV-1 replication via siRNA knockdown screen, and working on yeast two-hybrid (Y2H) detected the interaction between gHΔsp, gD baits and several CEACAMs preys. Specifically, CEACAM8 (in prey) interacted with gHΔsp (in bait) was confirmed. Furthermore, our recent result also indicated that several CEACAMs potentially enable to interact with either gB or gD (see “Result 5.2” and “Discussion 6.1.2”), which were the further evidences shown that CEACAM may serve as the cellular receptor for HSV-1 viral glycoproteins (gB/gD).

Interestingly, previous studies suggested that gH/gL act as the intermediate regulators between gB and gD to promote the fusion and as the carrier to load binding sites to the cellular receptors. Meanwhile, whether gH/gL could interact with a cellular receptor has not yet been identified (*Campadelli-Fiume et al., 2012*). However, our LUMIER data revealed the positive interaction between CEACAM proteins and gH (see “Result 5.2”) which fight against the preceding statement of a yet unidentified HSV-1 gH receptor. According to our LUMIER result, the g interaction between CEACAMs (CEACAM7, CEACAM8, CEACAM16, CEACAM19, CEACAM21) and gH can be seen obviously via the strong positive bars (see “Result 5.2”), this outstanding data demonstrated that CEACAM is likely to be the new receptor for gH, apart from the only known αVβ-intergrin routing factor.

Importantly, the cell species and the receptor classes may also influence the viral entry pathway. On the cell side, HSV-1 entry pathway differs in cell types, the direct endocytosis into the neutral endosome and virion-cell fusion at the plasma membrane are the common routes for the majority of cell species, whilst HSV-1 may also enter into some special cells via macropinocytosis (*Clement et al.,2006*).

On the receptor side, some routing factors such as αVβ-intergrin do not require receptor when shifting to the subdomain of the membrane. The endothelial strain of cytomegalovirus needs the special determinants of gH/gL for fusion (*Ryckman et al.,2008*).

6.3 The linkage between HSV-1 and Alzheimer's disease

Alzheimer's disease (AD) is a chronic and devastated neurodegenerative disease which the causes are poorly understood and the knowledge of cases are limited (*Miller & Federoff, 2008*). Studies showed that one factor of Alzheimer's disease is age, elder population over 65 years old are likely to be afflicted by AD, especially for the people over the age of 85 (*Piacentini,et a., 2014*). One recent clinical report indicated that nearly 35 million people worldwide were attacked by AD in 2010, and the number of population was dramatically increasing to approximately 60 million by the end of 2015, it is estimated that the peak will reach to 115-120 million by 2050 (*Taken from World Alzheimer's Report 2015*). The main symptom of Alzheimer's disease is dementia, the cognitive function of patients is reducing progressively, which leads to short-term memory loss. As time goes on, some more clinical manifestations come out followed by the disease advances, including motivation loss, lack of self-care ability, disorientation and language barrier (*Itzhaki et al., 1997; Piacentini,et al., 2014*). According to the previous studies, this disease process is mainly based on the cerebellar atrophy along with the decrement of cerebral function (*Porcellini, et al., 2010*).

Apart from the aging, there are several other risk factors for developing the alzheimer's disease, such as hypertension, lifestyle, emotional stimulation (depression) as well as genetic risk (*Imtiaz et al., 2014*). From the genetic point of view, the best known risk factor is the carriage of the ε4 allele of the apolipoprotein E (ApoE-ε4) which is capable of affecting neurodegeneration diseases (*Mahley et al., 2000*). The unique ApoE-ε4 plays a major role in AD pathogenesis. Meanwhile, ApoE-ε4 enables to increase the production of amyloid-beta (Aβ) peptide (the key component of senile plaques), and to stimulate the amyloid precursor protein (APP) (the cellular transmembrane glycoprotein and receptor), it also effects on the formation of the transgenic plaques (*Itzhaki et al., 1998; Holtzman et al., 2012; Verghese et al., 2013*).

Herpes simplex virus type 1 (HSV-1) DNA is a neurotropic virus which infects epithelial cells. As presented in the introduction chapter, primary HSV-1 infection

causes the ulcerative lesions such as cold sores. The acute infection undergoes the lytic replication, which the newly produced viral particles may migrate back to the original binding site via the sensory neurons, causing the recurrence. After the acute infection, some HSV-1 particles may conceal in sensory nerve for lifelong, which process is called the latent infection. The latent viruses typically persist in the trigeminal ganglia located in the brainstem, and these reactivated viral particles come from the repeated HSV-1 replication cycle may finally reach the central nervous system through blood stream and cause neurological disorders (*Burgos et al., 2005*).

One molecular study believed that the HSV-1 infected neuronal cells led to an intracellular enhancement of Aβ, and a decline of some APP fragments. This event showed the similar cellular results in relation to the AD development (*Wozniak et al., 2007; Chiara et al., 2010*). In addition, the dynamic interaction between HSV-1 capsid and APP facilitates the HSV-1 viral transport inside the infected cells (*Agostini et al., 2014*). Furthermore, ApoE-ε4 carrier has been confirmed to influence the establishment of latent infection and the expression of HSV-1 immediate-early genes using a HSV1-infected AopE-transgenic mice model (*Miller & Federoff, 2006*).

Genome wide association (GWA) study with several thousands of AD European patients demonstrated that few genes (such as Nectin2, CEACAM16, CEACAM17) and other host factors (HF) affecting HSV-1 infection were encoded close to the AopE region susceptibility locus on chromosome 19 were associated with late-onset Alzheimer's disease, and the single nucleotide polymorphisms (SNPs) in this region were linked to AD-associated SNPs (*Porcellini et al., 2010*).

According to several convincing studies, the hypothesis of the relationship between HSV-1 and Alzheimer's disease is potentially established, but further investigations are necessary in order to validate this standpoint.

6.4 Further work

The interaction between CEACAM proteins and viral glycoproteins has been detected via several independent repeating of LUMIER assay, but the positive control pair (either JUN/FOS or SPZ1/E7) was unstable based on the previous results, so choose a more efficient control pair will be much convincing. Alternatively, we could use HVEM receptor to interact with gD through LUMIER

simultaneously which the interaction has been confirmed, so that we could better confirm the interaction result.

Due to the result that CEACAM7 enabled to interact with gH, the further work in the laboratory will need to test whether other CEACAM proteins (such as CEACAM8, CEACAM16) could interact with gH by both immunoprecipitation and co-Immunoprecipitation assays. Meanwhile, we could test the downregulation of CEACAMs with overexpressed gH in mammalian cells through immunofluorescence (IF), which is a general biochemical application using confocal microscopy. Antibody plays an important role as probe, which aims to label the specific antigen with the staining marker fluorescein isothiocyanate (FITC) or DAPI (which can pass through the cell membrane) to target the molecule. The conjugation between the antibody and fluorophores makes the target protein visible under the microscope so that the distribution of proteins is obvious (*Bacallao et al., 2006; Robinson et al., 2009*).

Because of the results for the HSV-1 early entry event with CHO cells are variable, we could test other non-permissive cell lines such as Zebrafish, Drosophila which are suitable for infection. Also, we could test the CEACAM expression in CHO cells, HeLa cells or SK-N-SH cells by quantitative polymerase chain reaction (qPCR). Similarly, this technique, which aims to monitor an exponential amplification of targeted DNA, also rely on the fluorophore reporter. The accumulating DNA binds to the fluorescent dye and the value of fluorescence signal is recorded and the number of specific DNA section is doubled during each PCR process cycle, which depends on the change of temperature and the thermostability of DNA polymerase (*Higuchi et al., 1993; Lehmann & Kreipe, 2009*).

In addition, cell fusion assay is another approach to investigate whether CEACAM is a new cellular receptor, this technique has been applied to the research of other receptors, for example, 3-O HS is the receptor for gD and PILRα is the receptor for gB (see “Discussion 6.2”).

6.5 Conclusion

In this project, we found the significant increase of HSV-1 viral replication by overexpressing CEACAM proteins, and the interaction between several CEACAMs (such as CEACAM7, CEACAM8, CEACAM16, CEACAM19) and viral glycoproteins (gB, gD) was confirmed by LUMIER assay. Specifically, we also identified that CEACAM7 was able to interact with gH via Co-IP which fights against the previous

study said that the receptor for gH is unknow. The luciferase assay for HSV-1 early entry using viral non-permissive cell (CHO cells) indicated that CEACAM may work as the co-receptor with HVEM or Nectin, because CEACAM proteins obviously increased the susceptibility of HSV-1 infection and viral replication. Therefore, all the convincing results throw light on our hypothesis, which makes us more confident that CEACAM protein family represents a novel class of entry receptor for HSV-1.

7. Appendix

Appendix A:

Table 1: Materials of making medium and solution

Meidum / Solution	Materials
Pen-Strep	100units/ml Penicillin; 100µl/ml Streptomycin.
5% FCS+1% Pen-Strep	25ml FCS and 5ml P/S dissolved in 500ml DMEM
PBS (5X)	8.0g/L NaCl, 0.2g/L KCl, 1.42g/L Na2HPO4, 0.24g/L KH2PO4, PH=7.4
Trypsin	0.025% trypsin and 0.01% EDTA in PBS with phenol red
LB agar	1% (w/v) bacto-tryptone (Difco®), 1% (w/v) sodium chloride (Sigma-Aldrich®), 0.5% (w/v) yeast extract (Difco®), 1.5% (w/v) agar (Fluka®)
LB medium	1% (w/v) bacto-tryptone (Difco®), 1% (w/v) sodium chloride (NaCl; Sigma-Aldrich®), 0.5% (w/v) yeast extract (Difco®)
TAE buffer (1X)	0.04 M Tris, 0.001 M EDTA, 0.002 M glacial acetic acid
Lysis Part1 buffer	22mM Tris PH7.5, 1% TritionX100, 275mM NaCl, 11mM EDTA
1M DTT	1.5 g of DTT (DL-dithiothreitol) in 8 ml of deionized or distilled water, make up to 10 ml
1mM DTT	0.1M concentration of 1M DTT
Cell lyse buffer (LUMIER)	Lysis Part1 buffer 900µl, Phosphatase inhibitor cocktail (Roche® 04 906 837) 100µl, Protease inhibitor cocltail (Roche® 1 836 170) 40µl, 1M DTT 10µl, Benzonase (Novagen® 70746, puritiy>90%, 25 units/µl) 0.5µl (the total volume is for per 96-well plate)
luciferase activity measurement substrate	20ml of 1X PBS, 5M NaCl, 40µl aliquot of Coelenterazine
Citrate buffer	40mM Citric Acid, 135mM NaCl, 10mM KCl, PH=3.0
Passive Lysis Buffer (1X)	Dilute Passive Lysis Buffer 5X (Promega®) with water
NP40 lysis buffer	20mM Tris PH7.5, 150mM NaCl, 5mM $MgCl_2$, 1% NP40

10% Resolving gel	7.7ml ddH_2O, 4ml 40% Acrylamide, 4ml 1.5M Tris PH8.8, 160µl 10% SDS, 160µl 10% APS, 16µl TEMED
6% Stacking gel	7.05ml ddH_2O, 1.5ml 40% Acrylamide, 1.25ml 1.5M Tris PH6.8, 100µl 10% SDS, 100µl 10% APS, 10µl TEMED
Gel running buffer (10X)	Glycine 144.2g, Tris Base 30.3g, SDS 10g, make up to 1 litre with water
Gel transfer buffer (10X)	Glycine 29.3g, Tris Base 58.2g, SDS 3.7g, make up to 1 litre with water
TBS (1X)	6.05 g Tris and 8.76 g NaCl in 800 mL of H_2O. Adjust pH to 7.5 with 1 M HCl, make up to 1 litre with water
TBST (1X)	10% Tween 20 Solution in 1X TBS
5% milk of TBST (5% milk/TBST)	10g Marvel non-fat milk powder dissolved in 200ml TBST

Appendix B:

Table 2: GATEWAY plasmids

Plasmid	Alias	Antibiotic	Enzymes for restriction digest
pcDNA-RL-GW	pLUM	Ampicillin	XhoI/XbaI
pTREX-dest30-PrA	pTREX	Ampicillin	XhoI/NheI
pGBKT7	Bait	Kanamycin	EcoRI/BamHI
pGADT7-AD	Prey	Ampicillin	EcoRI/BamHI

Table 3: DNA plasmids in stock

Vector	**Gene which has been transformed into the plasmids**
pLUM	CEACAM1, CEACAM3, CEACAM5, CEACAM7, CEACAM8, CEACAM16, CEACAM19, CEACAM21; c-JUN
pTREX	CEACAM1, CEACAM3, CEACAM5, CEACAM7, CEACAM8, CEACAM16, CEACAM19, CEACAM21; c-FOS

Bait	CEACAM1, CEACAM3, CEACAM5, CEACAM7, CEACAM8, CEACAM16, CEACAM19, CEACAM21; c-MYC
Prey	CEACAM1, CEACAM3, CEACAM5, CEACAM7, CEACAM8, CEACAM16, CEACAM19, CEACAM21; MAX
pCR3	CEACAM1, CEACAM3, CEACAM5, CEACAM7, CEACAM8, CEACAM16, CEACAM19, CEACAM21, HVEM
pCDN3.1	Nectin

Table 4: Dilution of antibody in 5% milk/TBST (antibody:milk)

	IP	CO-IP
Primary antibody	Mouse α-MYC (1:2500)	Rat α-HA (1:2500)
Secondary antibody	α-mouse-HRP (1:5000)	α-rat-HRP (1:5000)

(For example, the ration of “1 : 2500” = the volume of “antibody : 5% milk/TBST”.)

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